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The *frpB1* gene of *Helicobacter pylori* is regulated by iron and encodes a membrane protein capable of binding haem and haemoglobin

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ABSTRACT

FrpB1 is a novel membrane protein of *Helicobacter pylori* that is capable of binding both haem and haemoglobin but consistently shows more affinity for haem. The mRNA levels of *frpB1* were repressed by iron and lightly modulated by haem or haemoglobin. The overexpression of the *frpB1* gene supported cellular growth when haem or haemoglobin were supplied as the only iron source. Three-dimensional modelling revealed the presence of motifs necessary to bind either haem or haemoglobin. Our overall results support the idea that FrpB1 is a membrane protein of *H. pylori* that allows this pathogen to survive in the human stomach.

1. Introduction

Haem is an available iron source in humans and is sequestered by proteins, such as haemoglobin (Hb) or haemopexin. Hb is potentially abundant in humans and forms the majority of the circulating haem supply [1]. Therefore, such sources are attractive to pathogens because they can access them in many tissues during infection to meet their iron needs [2,3]. To use haem or Hb, bacterial pathogens have developed sophisticated mechanisms to obtain iron from these sources, which consist of secreted soluble proteins named haemophores that transport the iron source to a membrane receptor [4]. The iron source is then introduced into the bacterial cytoplasm via a Ton B-dependent mechanism [5]. It has been proposed that all these proteins bind the iron source via two motifs (FRAP and NPNL) [6]. Although these receptor proteins have been identified in several bacteria, including *Escherichia coli* ChuA (69.5 kDa) [7] or *Corynebacterium diphtheria* (24.1 kDa) [8], there are bacteria that possess more than one receptor. For instance, *Pseudomonas aeruginosa* expresses PhuR and HasR, and both receptors are capable of binding haem and Hb [9]. In addition, *Helicobacter pylori* expresses three haem-binding outer membrane proteins, with sizes of approximately 48, 50 and 77 kDa, but their respective identities remain unknown [10]. Recently, another protein termed FrpB2 (90.8 kDa) was identified in *H. pylori*. This protein binds Hb and is located in the membrane [11]. In addition, microarrays assays showed that under iron starvation resulted in a 10-fold increase, the transcript levels of gene, *frpB1* encoding a hypothetical iron-regulated outer membrane protein FrpB1. However, its function remains undetermined [12]. As there is no evidence that *H. pylori* secretes haemophores to scavenge haem or Hb and deliver it to a membrane receptor, it is tempting to speculate that this pathogen must be equipped with more membrane proteins that directly bind these iron sources, as proposed by Worst et al. [13]. However, it is not feasible to study these genes of *H. pylori* by mutagenesis, because a deletion could be easily compensated for by another gene or pathway, such as in *Haemophilus influenzae* [13]. Accordingly, we present a strategy that allows us to suggest that FrpB1 is a new haem- and Hb-binding protein with characteristics different from those reported previously for other proteins. It is possible that the FrpB1 protein participates in a more sophisticated mechanism that has developed in *H. pylori*, which is necessary to confront the extremely acidic environment of the stomach.

2. Materials and methods

2.1. Quantification of frpB1 mRNA levels

Total RNA of *H. pylori* growing under conditions: iron replete, iron absence (medium was supplemented with 2,2′-dipyridyl),

Abbreviations: csp, cold shock protein; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; K_d, dissociation constant; NaH_2PO_4, monobasic sodium phosphate; PBS, phosphate-buffered saline; SD, standard deviation; UV/VIS, ultraviolet/visible

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and haem or Hb used as the sole iron source was purified using the phenol/chloroform technique [14]. Semi-quantitative RT-PCR was performed using 10 ng of total RNA, SuperScript One-Step (Cat. 11922028, Invitrogen) and specific oligonucleotides for *frpB1* (forward, 5′-ACTAAAGGCGCGGCGAATGCGAGCGC-3′, and reverse, 5′-GGACACCCTTGAGCGCCATCAGGGCT-3′). The primers were designed according to the annotated sequence in PubMed (HP0876). The RT-PCR conditions for the *frpB1* gene were as follows: 45 °C for 30 min, 94 °C for 2 min, 23 cycles (at 94 °C for 1 min, 50 °C for 1.30 min, and 72 °C for 1.20 min), and a final extension step at 72 °C for 10 min. As a positive control, a reaction using oligonucleotides that amplify 235 bp of the 23s mRNA was performed. A reaction was prepared without reverse transcriptase as a negative control. The amplicons were separated on 1% agarose gels, which were stained with ethidium bromide and subjected to densitometric analysis using Quantity One-4.6.3 1-D Analysis Software. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, (www.graphpad.com).

2.2. Cloning of the frpB1 and chuA genes

The *frpB1* gene was amplified from *H. pylori* genomic DNA by PCR using the primers, 5′-GGGGGGAGCTCATGTTTTTAAGATCATACCCAAAGCTTAGATA-3′ (forward) and 5′-GGGGGGAATTCCTACCATTTGTAAGCCACTTCAAAACG-3′ (reverse), designed according to annotated sequences (GeneID NCBI accession number 890085). The *chuA* gene used in this work as a positive control was amplified from *E. coli* (strain *EC EH O157:H7 EDL 933*) genomic DNA using the primers, 5′-GGGGGGAGCTCATGTCACGTCCGCAATTTACCTCGTTGCGTTTGAGTTT-3′ (forward) and 5′- GGGGGGAATTCTTACCATTGATAACTCACGAAAATTTTTCCGTTACGACCATC-3′ (reverse) (GeneID NCBI accession number AAG58641), designed according to an in silico analysis. Both of the PCR products were inserted in pColdI DNA (Cold-Shock expression vector, Takara) using the *Sac*I and *Eco*RI restriction sites. The identity of each gene was corroborated by sequencing analysis.

2.3. Expression of FrpB1 and ChuA proteins in E. coli

E. coli (BL21pLys) competent cells transformed with pCold-frpB1 or pCold-chuA were cultivated in Luria–Bertani medium to 0.4 O.D. Gene expression was induced by adding 1 mM IPTG, and the culture was incubated at 30 °C. The cells were then collected by centrifugation (8000×g), and the pellet was suspended in PBS plus lysozyme (1 mg/ml). Aliquots of 1 mM PMSF and Triton X-100 were added to a final concentration of 1%. The cells were broken by sonication at 4 °C for 2 min, using 20-s pulses and 60% amplitude. The samples were then centrifuged at 1500 X g for 5 min. The supernatant was ultracentrifuged at 105 000×g for 1 h to obtain soluble and insoluble fractions. The insoluble fraction, considered as a source of membrane proteins, was suspended in PBS containing 1 mM PMSF and 1% Triton X-100. The samples were then loaded onto a Nickel-Sepharose High Performance column (Qiagen). The flow-through fraction was collected, and non-specific interactions were eliminated by washing the resin three times with wash buffer (containing 50 mM NaH_2PO_4 and 300 mM NaCl) and 50, 65, and 80 mM imidazole. The proteins were released with pH 8.0 elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole), and each fraction was analysed using Coomassie blue-stained SDS–PAGE. The presence of the FrpB1 and ChuA proteins was corroborated using Western blot analysis using anti-His antibodies.

2.4. Overlay assay

The purified proteins (FrpB1 and ChuA) separated by 10% SDS–PAGE were transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk in PBST buffer (PBS, pH 6.8, and 0.05% Tween 20). Hb (10 mM iron) was added and incubated for 12 h at 4 °C. After three washes (15 min) with PBST, the membrane was incubated with anti-β-globin antibodies. Lastly, the secondary antibody coupled to horseradish peroxidase was added, and the signal was developed using chemiluminescence (Millipore) [15].

2.5. Haem-binding assay

Binding assays were performed to test the ability of the FrpB1 protein to bind haem. Purified protein (20 μM) was suspended in binding buffer (250 mM Tris, 5 mM EDTA, 10% glycerol and 20 μM haem, pH 8.0), and the samples were incubated at 37 °C for 30 min and loaded onto 7% native PAGE. Proteins were visualised by Coomassie blue-staining, and the haem interaction was revealed by the hydrogen peroxide technique (a brown colour is observed in the gel) [16].

2.6. Haem and Hb assays to estimate the binding affinity

To estimate the affinity of the FrpB1 and ChuA proteins for haem or Hb, binding assays were performed using UV–VIS spectrophotometry. Purified proteins (20 μM) in binding buffer were titrated with increasing concentrations of haem or Hb (1, 2, 7, 15, 46, 77, 150, 310, 460, 610, 770, and 930 μM). The absorbance in the UV–VIS spectrum between 200 and 900 nm was scanned using a spectrophotometer (Supplementary data Figs. 10 and 11, supplementary material). The spectra were recorded 1 min after the addition of each haem or Hb aliquot in triplicate in three separate experiments. The intensities of the Soret peaks at 238 nm of the haem-FrpB1 (Fig. 10B), at 451 nm of Hb-FrpB1 (Fig. 10C), at 245 nm of haem-ChuA (Fig. 11B) and at 456 nm of Hb-ChuA (Fig. 11C) were evaluated to monitor the complex formation. To generate the binding isotherms, the absorbance values were plotted against the haem or Hb molar concentration (Supplementary data). The data were fitted to a one binding-site model using the non-linear regression function to determine the dissociation constant (K_d), assuming that binding follows the law of mass action (Supplementary data). Statistical analyses, binding stoichiometry and affinity estimations were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, (www.graphpad.com).

2.7. Media and cultures

It has been documented previously that ingredients in the culture medium, including peptone or tryptone, can block the uptake of Hb or haem. For this reason, the cells were cultured in M63 minimal medium supplemented with ferric citrate [17]. *E. coli* transformed with the pCold, pCold-frpB1 or pCold-chuA plasmids were inoculated on plates containing 250 μM 2,2′-dipyridyl (Sigma–Aldrich), 1 mM IPTG (Fermentas, Life Sciences) or supplemented with equivalent molar quantities (10 mM iron) of haem or Hb (Table 1) and incubated at 30 °C for 28 h. In the case in which growth cell was observed, the diameter of colonies was measured (diameters oscillating between 2 and 3 mm were observed). Positive cellular growth was indicated as (✓) and negative (×) in which no colonies were observed.

3. Results

3.1. The expression of the frpB1 gene is increased in H. pylori under iron absence conditions and slightly modulated by the supply of haem or Hb

It is known that the mRNA levels of the *frpB1* gene are increased under iron-limiting conditions [12]; therefore, other iron sources,

Table 1
The expression of the *frpB1* gene supports the growth of *E. coli* in minimal media supplemented with ferric citrate. When required 2, 2′dipyridyl or IPTG were added, Hb or haem were utilised as the sole iron source. Positive cellular growth (✓) when colonies were observed in the plate (diameters fluctuated between 2–3 mm), Negative cellular growth (×) no colonies were observed.

	M63	IPTG	2,2′-Dipyridyl	Haem	Hb	Cellular growth
pCold	✓	×	×	×	×	✓
pCold	✓	✓	×	×	×	✓
pCold	✓	×	✓	×	×	×
pCold	✓	✓	✓	✓	×	×
pCold	✓	✓	✓	×	✓	×
pCold-frpB1	✓	×	×	×	×	✓
pCold-frpB1	✓	✓	×	×	×	✓
pCold-frpB1	✓	×	✓	×	×	×
pCold-frpB1	✓	✓	✓	✓	×	✓
pCold-frpB1	✓	✓	✓	×	✓	✓
pCold-chuA	✓	×	×	×	×	✓
pCold-chuA	✓	✓	×	×	×	✓
pCold-chuA	✓	×	✓	×	×	×
pCold-chuA	✓	✓	✓	✓	×	✓
pCold-chuA	✓	✓	✓	×	✓	✓

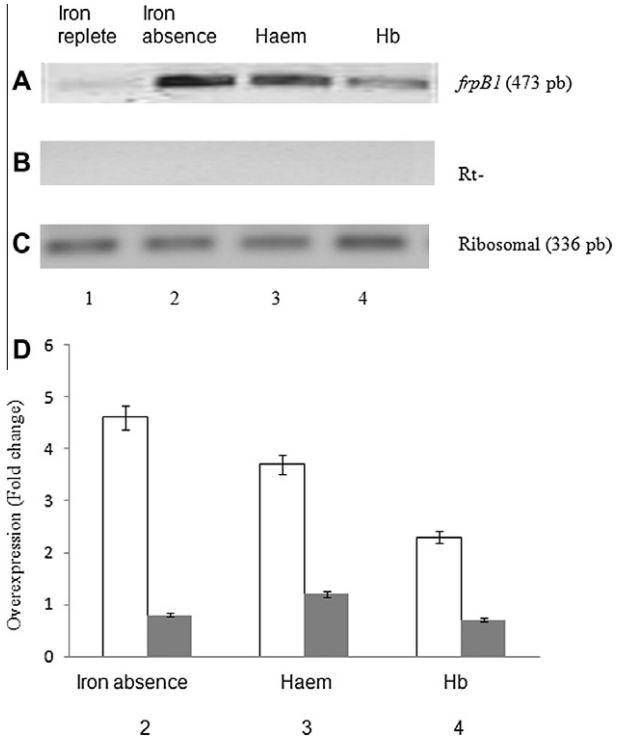

Fig. 1. *H. pylori* expresses *frpB1* under different iron conditions. Total mRNA of *H. pylori* from cultures grown under iron restriction (2,2′-dipyridyl added) (lane 2), with iron (lane 1), with either haem (lane 3) or Hb (lane 4) were collected. The mRNA samples were assessed using semiquantitative RT-PCR. The reaction products were loaded onto a 1% agarose gel and stained with ethidium bromide. Each band was subjected to densitometry analysis to quantify the gene expression. The plot shows the fold change considering the data obtained under iron-replete conditions (lane 1) as 1. White bars (*frpB1* expression); grey bars (constitutive gene expression). Error bars are shown in order to indicate the dispersion of the data (SD).

such as haem or Hb could also affect the expression of this gene. Our results showed high mRNA levels of *frpB1* when *H. pylori* was grown under iron depletion (medium was supplemented with 2,2′-dipyridyl) (Fig. 1A, lane 2) but not when iron was available (Fig. 1A, lane 1). Interestingly, the presence of haem or Hb (Fig. 1A, lanes 3 and 4) in the medium also increased the *frpB1* mRNA expression; nevertheless, the levels never achieved those obtained under the absence of iron. Perhaps the haem and Hb provide the intracellular iron that is necessary to modulate gene

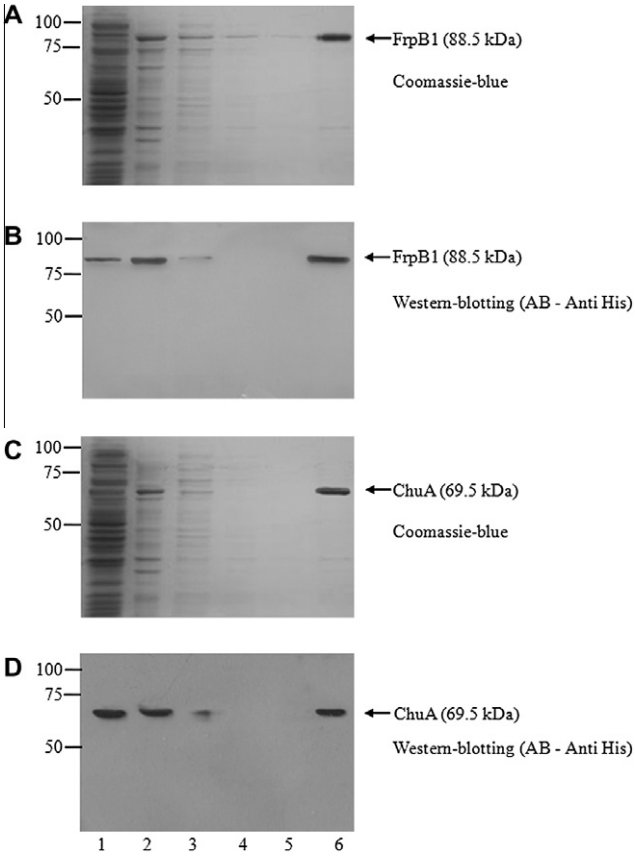

Fig. 2. Purification of FrpB1 and ChuA proteins by affinity chromatography. The total membrane fraction (lane 1) of *E. coli* transformed with the pCold-frpB1 (A) or pCold-chuA (C) plasmids were loaded onto nickel affinity chromatography columns, and the flow-through (lane 2) and washes (lanes 3–5) were collected. FrpB1 protein was eluted (lane 6). (C) ChuA protein was purified in the same way. The FrpB1 and ChuA identities were corroborated using Western blot analysis with anti-His antibodies (B and D).

expression. A reaction lacking RT was performed as a negative control (Fig. 1B), and the ribosomal gene (Fig. 1C) was included as a positive control. The plot shows the fold change considering the data obtained under iron-replete conditions (lane 1) as 1. These results indicate that *frpB1* gene expression depend on an iron source.

3.2. FrpB1 is an Hb-binding protein

The fact that the *frpB1* mRNA levels were partially repressed by Hb and haem suggested that *frpB1* encodes a protein involved in the uptake of these molecules. To explore this assumption, the *frpB1* gene was cloned and expressed, and the recombinant protein was purified. Total membrane proteins (Fig. 2A, lane 1) were loaded onto nickel affinity chromatography (Fig. 2A, lane 6); after three washes (Fig. 2A, lanes 3–5), purified FrpB1 was eluted (Fig. 2A, lane 6). The FrpB1 identity was corroborated using Western blot analysis using anti-His antibodies (Fig. 2B, lanes 1–6). We utilised ChuA (from enterohaemorrhagic *E. coli*) as a positive control because it has the ability to bind both Hb and haem sources [7]; this recombinant protein was obtained in the same way as FrpB1 was obtained (Fig. 2C and D). Subsequently, the Hb-binding capacity of both of the purified proteins was determined using an overlay assay (Fig. 3A, lanes 1 and 2). No detection of Hb-binding was observed with the GST protein used as a negative control (Fig. 3A, lane 3) [15,16] or when the overlay assay was performed without Hb (Fig. 3B). These findings further suggest that the FrpB1 protein is involved in Hb-binding.

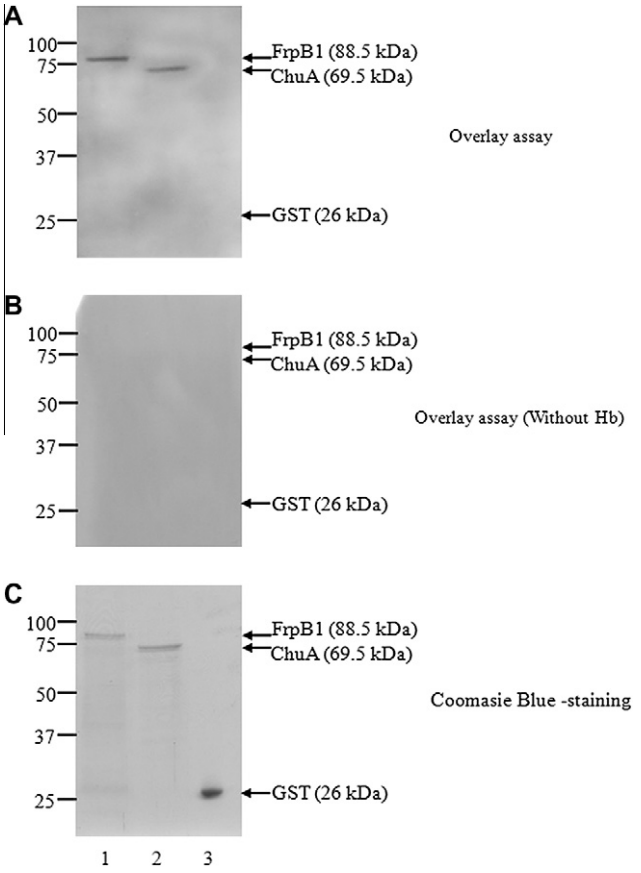

Fig. 3. Similar to ChuA, the FrpB1 protein binds human Hb. The Hb-binding capacity of the purified FrpB1 and ChuA proteins was analysed using an overlay assay. (A) Overlay assay for FrpB1 (lane 1) and for ChuA (lane 2); GST protein was used as an unrelated protein (lane 3). (B) Overlay assay was performed without Hb (negative control). (C) SDS–PAGE Coomassie blue staining. FrpB1, ChuA and GST purified proteins are indicated with arrows.

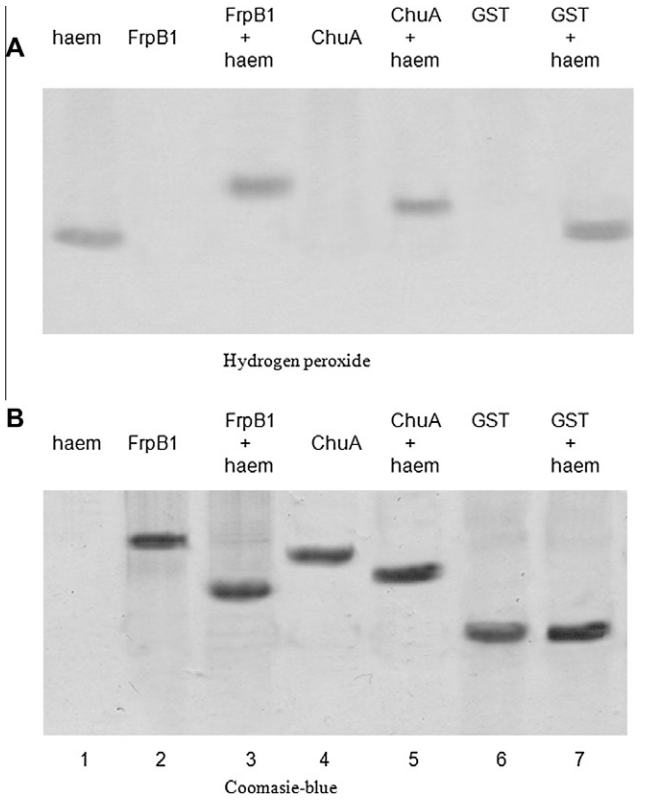

Fig. 4. Similar to ChuA, the FrpB1 protein is capable of binding haem. Purified proteins were suspended in binding buffer, and haem was added. (A) After incubation, the samples were loaded onto native PAGE gels, which were stained by hydrogen peroxide to detect the haem. (B) As in A, but the gel was stained with Coomassie blue to reveal the proteins. An electrophoretic migration for haem (lane 1), FrpB1 (lane 2), ChuA protein used as a positive control (lane 4), and GST used as an unrelated protein (lane 6). Complex formation between FrpB1 and haem (lane 3), ChuA protein and haem (lane 5), and the mixture of GST and haem in which no complex is formed (lane 7).

3.3. FrpB1 protein binds haem

Because the mRNA levels of the *frpB1* gene were modulated by the presence of haem, we speculated that FrpB1 could be a haem-binding protein. Our results showed that FrpB1 was capable of binding haem (Fig. 4A and B, lane 3), and a shift in the mobility of haem was observed when FrpB1 was present in the reaction (Fig. 4A, lane 3). A similar result was obtained with ChuA (Fig. 4A and B, lane 5). When an unrelated protein (GST) was analysed, no changes in the mobility were detected (Fig. 4A and B, lane 7). This result clearly indicates that FrpB1 can bind haem in addition to Hb.

3.4. Unlike ChuA, FrpB1 has a preference for haem

Although FrpB1 binds both haem and Hb, the binding behaviour might be different for each iron source. To explore this assumption, we calculated the K_d for FrpB1 and for the positive control (ChuA) using Hb or haem as the ligand (as described in the Section 2). K_d calculations indicate that FrpB1 has more affinity for haem (1.5 μM) than for Hb (10 μM). Conversely, the affinity of the ChuA protein was 1.6 μM for haem and 1.0 μM for Hb (plots are included in the Supplementary data). We speculate that the FrpB1 protein is synthesised by *H. pylori* to bind both sources but has a preference for haem.

3.5. The overexpression of *frpB1* in *E. coli* lacking Hb-binding genes allows cellular growth when haem or Hb are supplied as the only iron source

To explore the functionality of the FrpB1 protein further, we analysed whether the *frpB1* gene overexpressed in a heterologous system allowed haem or Hb to be utilised. Our results showed that *frpB1* and *chuA* (Table 1) overexpressed in *E. coli* permitted cellular growth when haem or Hb were added to the medium as the only iron supply. If the genes were not induced (Table 1) or the medium was not supplied with Hb or haem, the cells were incapable of maintaining growth. To eliminate the possibility that the presence of enterobactin (a siderophore secreted by *E. coli* to scavenge iron) might be helping to support the observed cellular growth, a control was performed using cells transformed with the vector (pCold) and were cultivated without iron (adding 2,2-dipyridyl). Under these conditions, no cellular growth was observed, indicating that enterobactin did not participate in this process. These findings suggest that, similar to the *chuA* gene, the *frpB1* gene encodes a protein involved in iron utilisation of haem and Hb sources.

4. Discussion

In this study, the participation of FrpB1 in iron uptake was explored. FrpB1 was characterised as a haem- and Hb-binding protein located in the membrane. As FrpB2 has previously been

described as an Hb-binding protein [11], we propose that FrpB1 is a novel protein capable of binding both haem and Hb iron sources. It is possible that this protein may participate in different mechanisms because FrpB1 is expressed under iron starvation, whereas the transcript of FrpB2 is not detected [12]. The molecular weight was estimated as 88.5 kDa, which is smaller than FrpB2 [11]. This finding is another distinction that suggests that these proteins have a particular role in the biology of *H. pylori*. We suggest that the *frpB1* gene is involved in iron acquisition from haem or Hb because this gene is able to maintain the growth of *E. coli* in the presence of these iron sources, whereas FrpB2 can only bind and use Hb [11]. The presence of this protein in the membrane fraction of *E. coli* suggested that FrpB1 could be a haem or Hb receptor protein, which is another characteristic of these molecules [9]. This assumption was supported by a comparison of the 3D structure (Supplementary data), in which, the structure was very similar to ChuA, the FRAP/NPNL motifs were conformationally conserved, suggesting that both proteins FrpB1 and ChuA could have similar roles in their respective organisms. Our overall findings indicate that *H. pylori* possesses a battery of genes, as proposed Worst [10], which are differentially regulated to confront the extreme conditions in the host. Perhaps *frpB1* is one of the first genes of this machinery that is expressed under iron absence; to bind Hb or haem released from ulcers caused by this pathogen. Using this via *H. pylori* could achieve its levels of iron necessary to survive in the human stomach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.015.

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